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BIOLOGICAL BULLETIN

SUPPLEMENTARY NOTES ON TWINS IN CATTLE.

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Since the publication of my previous papers on the free-martin (1916, 1917) more material has been slowly accumulating. The additional data have confirmed and strengthened my former conclusions, and they also include some observations of a certain amount of intrinsic interest. The present paper, therefore, is a series of supplementary notes on twinning in cattle, with comments on more recent literature.

1. CASES WHERE THE FEMALE OF TWO-SEXED PAIRS IS NORMAL.

In my previous paper I concluded that the free-martin is zygotically female, and that its intersexual condition is due to action of the blood of the male twin through anastomosis of the fœtal blood vessels which develops very early; Keller and Tandler (1916) came to the same conclusion as the result of entirely independent studies. This theory receives a crucial test in those relatively rare cases of two-sexed twins in which no such anastomosis develops, for in such cases the reproductive system of the female should be normal. In my previous paper I reported three cases of a normal female twinned with a male out of twenty-four pairs of two-sexed twins. The records of the first two cases (Nos. 8 and 9) were, however, incomplete, and the question of vascular anastomosis was not fully investigated because they were received before any theory on the subject was developed. They were, however, consistent with the above interpretation, for it was recorded in the notes made at the time of collection that the connection between the two chorions was narrow; presumably there was no vascular anastomosis. The third case (No. 40) was, however, a perfect test case in every respect; there was a corpus luteum in each

maternal ovary; the two chorions were entirely separate and there was, therefore, no question of vascular anastomosis.

Since this publication two additional cases have been received (Nos. 63 and 64) similar in every respect to case 40. The chorions were entirely separate in these cases also, and in each pair both the male and female possessed normal reproductive organs. In a third case, not previously reported (No. 93), in which the female was also normal, the chorions were connected, but there was no vascular union between the two sides. The notebook records of these cases follow:

No. 63. December 19, 1917; twins in uterus; mother's ovaries missing. Uterus carefully opened; the membranes appear at first sight to be fused in the body of the uterus; but they were really entirely separate, the end of one being merely slightly invaginated over the other, so that they fall apart intact. Length of male and female 22 cm. each; reproductive organs of both entirely normal.

No. 64. February 1, 1918. Twins in uterus; both mother's ovaries present; corpus luteum in each. Uterus carefully opened and chorions found to be entirely separate. In this case the openings from the horns into the body of the uterus were extremely constricted and the chorions were confined to the horns. Male 14 cm. long; female 13.75 cm. long. Reproductive organs of both entirely normal.

No. 93. December 1, 1920. The twins were in the intact uterus; mother's ovaries present; a corpus luteum in each ovary. The chorions when exposed were found to have a very narrow connection, which on examination turned out to be non-vascular. Length of male twin 10.25 cm., of female 9.75 cm. Reproductive organs of both entirely normal.

Keller and Tandler (1916) report six cases, out of a total of 91 cases of two-sexed twins of cattle in which the fœtal membranes were examined, in which the female was normal. In one of these the two chorions were adherent, but not fused in any way; in another there was a narrow non-vascular strand uniting the two chorions apparently similar to my case No. 93. In the remaining four cases the chorions were well united, but the place of fusion was indicated by a narrow strip of white, scar-like tissue completely encircling the chorion; in three of these cases no vascular anastomosis could be demonstrated across the place of fusion; in the fourth case several exceedingly small blood vessels crossed, very different from the typical wide connection between the two circulations. The last case was in the fourth month of pregnancy (size of fœtuses not given). The connections in question may

have failed on account of their small size, but it seems more probable that they represented secondary connections formed too late to be effective.

We thus have twelve cases on record in which the female of two-sexed twins of cattle is normal, and in which the conditions of the fœtal membranes are also known—six cases of Keller and Tandler and six cases of my own. In nine of these cases there was certainly no vascular connection; two of my own cases were not satisfactorily investigated with reference to this point, and in one of Keller and Tandler's cases the fine anastomoses that existed were apparently secondary. In all other cases of two-sexed calf twins in which the fœtal blood vessels were examined (29 of the author and 85 of Keller and Tandler) there was anastomosis between the fœtal vessels and the female was definitely intersexual.

The proportion of cases in which the female of two-sexed twins in cattle is normal was found by Keller and Tandler to be 6 out of 91; in my collection it is 6 out of 39; Numan estimates the proportion to be 1 in 8, and Lüer (1913) 6 in 113. (Cited from Keller and Tandler.)

The experiments of Minoura (1921) in which he grafted pieces of testis or of ovary on the allantoic membrane of chick embryos and obtained as a result intersexes of various grades, furnish complete proof that sex hormones can produce precisely the same kind of changes that are found in the free-martin.

2. The Sex-ratios.

The original account involved 54 cases of fœtal twin calves in which the distribution of the sexes was as follows: ♂ ♂ 19, ♂ ♀ 3, ♂ ☿ 21, ♀ ♀ 11; or classifying the normal and intersexual females of two-sexed pairs together, ♂ ♂ 19, ♂ ♀ 24, ♀ ♀ 11. The expected ratios, assuming approximate equality of male and female zygotes in cattle, would be in the proportions of 1 : 2 : 1—*i.e.*, 13.5 : 27 : 13.5. It is obvious that there is an excess of male pairs which is not readily interpretable as due to chance, even though the numbers are relatively small.

I have now 38 additional pairs of fœtal twin calves (Nos. 58–97, excluding No. 66, which was a pair selected after birth, and No. 80,

which was apparently monozygotic); the addition of these gives the following distribution of the sexes for all my cases: ♂♂ 29, ♂♀ 6, ☿♂ 33, ♀♀ 24; or again combining normal and intersexual females of two-sexed pairs together: ♂♂ 29, ♂♀ 39, ♀♀ 24. Assuming an equality of the sexes in the population dealt with, the expected distribution of sex in the 92 pairs of twins would be ♂♂ 23, ♂♀ 46, ♀♀ 23. It is obvious that there is an excess of male pairs and a deficiency of two-sexed pairs on this assumption. The numbers are not very large, but probably sufficiently so to be significant.

The aberrant ratio for the twins would be satisfactorily explained if the transformation of the free-martin sometimes went so far in the male direction as to render it indistinguishable from a male. I have, therefore, recently studied male pairs with considerable care, but have found no evidences of abnormalities in the reproductive organs; in my experience the structural differences that distinguish a free-martin from a male are always very great. It was natural to expect the greatest modification of the female when both the male and female twin came from the same ovary and developed in the same horn of the uterus, for under these circumstances the vascular anastomosis would presumably be unusually early and extensive. The two cases of this sort which I have, however, show no greater modification of the female than many others (see Sec. 4).

It appeared probable, therefore, that the assumption of approximate numerical equality of the sexes in the population was at fault. I therefore suggested to Mr. J. M. Jewell that he ascertain the sex-ratio in fœtal calves taken from the same slaughter-house. Accordingly the sex-ratio of 1,000 cases taken at random was determined (Jewell, 1921). For our purpose it is sufficient to notice that a sex-ratio of 123.21 was established for the 1,000 cases, including all fœtal ages; the sex-ratio for the sizes included in the twin collection (3.5–30 cm. in length) was found to be 120 (*i.e.*, 120 males for every 100 females; 264 entries).

Taking this figure as the sex-ratio of the population from which the twins came, the expectation of sex distribution in the 92 pairs of twins would be ♂♂ 27.37, ♂♀ 45.62, ♀♀ 19.01. This shows a type of sex distribution similar to that actually found in the twins,

which, however, show a sex-ratio of only 111.5 counted as individuals. The approximation of the twin sex-ratio to the expected sex-ratio is, therefore, sufficiently exact when the smallness of numbers is taken into account to exclude the need of any special assumption.

Incidentally it may be noted what a complete demonstration the figures furnish of the underlying assumption that the free-martin is zygotically female, which D. Berry Hart apparently continues to question (1918). In a recent paper Gowen (1921) presents extensive data showing that twins of cattle, whether of the same sex or both, do not resemble one another in color more than full sisters of the same breed. As the latter are, of course, dizygotic, the presumption is that the twins are so likewise, or at least that monozygotic twins in cattle are extremely rare.

3. On Monozygotic Twinning in Cattle.

The embryological criterion for distinguishing dizygotic and monozygotic twinning in cattle would be the occurrence of two corpora lutea in the maternal ovaries in the former case and of one in the latter. Implantation of the fœtuses in one horn of the uterus would also be expected, but this can not be relied upon by itself as evidence, for one ovary often furnishes two ova, both usually remaining in the horn of the uterus on the same side as the ovary. It is also conceivable that two ova derived one from each ovary might lodge in one horn of the uterus owing to passage of one of them from one horn to the other; however, I have found no such case. The existence of a single chorion would also, of course, be postulated in monozygotic twinning, but its value as evidence would be negligible owing to the tendency for originally separate chorions to fuse in cattle.

In the case of cattle, therefore, the embryological evidence for monozygotic twinning is reduced to the occurrence of only one corpus luteum for two embryos of the same sex. One such case (No. 80) occurs in my collection. The chorion is single, resembling in all respects cases in which the usual two corpora lutea are from one ovary; the amniotic cavities were adjacent, but not communicating; the twins were females of about 16 cm. greatest length. The preparation has been preserved complete in formalin

to demonstrate these points. This may be a case of monozygotic twinning; but the possibility that the Graafian follicle furnishing the corpus luteum contained two ova, both of which were fertilized and developed, can not be neglected.

My records include 36 cases of twins in which both maternal ovaries were present; of these 35 had two corpora lutea either in both ovaries or in one. In the 90 cases reported by Keller and Tandler there were 88 cases of two corpora lutea and 2 of three corpora lutea. Thus only one case of 126 known cases exhibits one corpus luteum for two embryos. These statistics demonstrate how futile it is to continue to appeal to monozygotic twinning, as D. Berry Hart (1918) does, as the cause of the intersexual condition of the free-martin.

4. The Formation of the Free-martin an "All-or-none" Reaction.

Cases of two corpora lutea in one maternal ovary are apparently not very rare in cattle twins, to judge from the statistics of Keller and Tandler, who found 36 such cases out of 90. I have found only three such cases out of 36 cases where both ovaries were present, but the small number may be due to the fact that the butchers, on whom we relied for selecting uteri containing twins, would be likely to overlook twin pregnancies confined to one horn of the uterus. Cases of this kind in which one fœtus is a male and the other a free-martin are of considerable theoretical interest, because implantation of the twins in one horn of the uterus offers presumably the maximum opportunity for modification of the female.

In all cases in which the twins come from two ovaries it is necessary for the chorionic vesicles to grow entirely through their respective horns of the uterus before they can meet in the corpus uteri, and the fusion that takes place there must occur near the apices of the vesicles, as far removed from the embryos as possible. It is probable that the embryos would be about 15 mm. long before fusion (cf. my earlier paper, p. 389), and we have no data for deciding how quickly vascular anastomosis would become established and effective. When, on the other hand, both ova come from one ovary (Fig. 1) and develop in one horn of the uterus,

FIG. 1. Case No. 72. Female twins. To illustrate the membranes when both embryos are derived from one ovary. Two corpora lutea occur in ovary 1, none in ovary 2. Both embryos were in one horn of the uterus. Each 28.5 cm. long. *A. P.* shows the position of the partition between the two amniotic sacs. An arterial anastomosis is shown in the insert figure which shows the inner surface of the cotyledon at *X* between the points *A* and *B* of the main figure. (× by 5/22.)

FIG. 2. The membranes of Case 78 described in the text to show the relations of the two amniotic sacs and the strong anastomosis between the umbilical arteries of the twins (× by about ¼). Embryos 32 cm. long.

fusion may take place at a much earlier stage; and vascular anastomosis may date from the time of extra-embryonic circulation. The approximation of the embryos in the common chorion may give some indications of the manner and time of fusion, and the vascular relations will also throw some light on the same questions.

I have two cases of this kind (Nos. 78 and 97) in which the fœtuses are of opposite sex, but in neither case is the modification of the free-martin particularly extreme. They are so alike that the description of one of them (No. 78) will suffice. Fig. 2 is from an oil painting showing the membranes; the very direct arterial connection between the two umbilical cords will be noted. The two amniotic cavities are so close together that the walls are in contact at one place. The fusion of the two original vesicles was presumably, therefore, side by side and very early, for all overlapping parts have disappeared without leaving any trace.

If I were to venture to reconstruct the probable history of this case, I would say that the fusion was probably complete at least by the 10-mm. stage, and vascular anastomosis established at the same time. The reasons for this opinion are the extent to which the membranes of a 10-mm. embryo fill up one horn of the uterus (see Lillie, 1917, p. 389) and the readiness with which fusion of the membranes takes place (specifically in cattle) when they are in contact. If this opinion is correct, the vascular anastomosis in this case dates from long before the period of beginning of sex differentiation.

The anatomy of the reproductive organs of the free-martin is shown in Figs. 3*A* and 3*B*. If these figures be compared with figures of free-martin anatomy given in my 1917 paper and with Figs. 4 and 5, it will be seen that the extent of the modification is less than the average in certain respects. Thus the cornua and corpus uteri are retained in this case, though very rudimentary compared with the normal; in probably the majority of free-martins they are entirely absent (cf. Fig. 5); the Wolffian ducts are also more slender than in most free-martins, and the gubernacula less typically developed (cf. Fig. 4). The gonads are exceptionally minute, which would appear to indicate less than the average stimulation of the male homologues in the original ovary

FIG. 2. The membranes of the twins (× by about ⅓). Embryos 32 cm. long. the umbilical arteries of the twins

out of which the free-martin gonad is entirely composed (cf. Chapin, 1917; Willier, 1921).

FIG. 3. Anatomy of the urinogenital system of the free-martin of Case 78, 32 cm. long. Described in the text. Greatest length, 32 cm. *A*. The entire system. *B*. The urinogenital cord has been cut across in front of the seminal vesicles and turned over anteriorly so as to expose the dorsal surface. 1. The exceedingly minute gonad; 2. Cornua uteri; 3. Gubernacula invaginated into the body cavity; 4. Cut end of umbilical artery; 5. Wolffian ducts; 6. Very slender seminal vesicles. Natural size.

The idea that I have expressed elsewhere (1917) that the variable degree of intersexuality exhibited by free-martins may be a function of the time of onset, intensity, and perhaps duration of

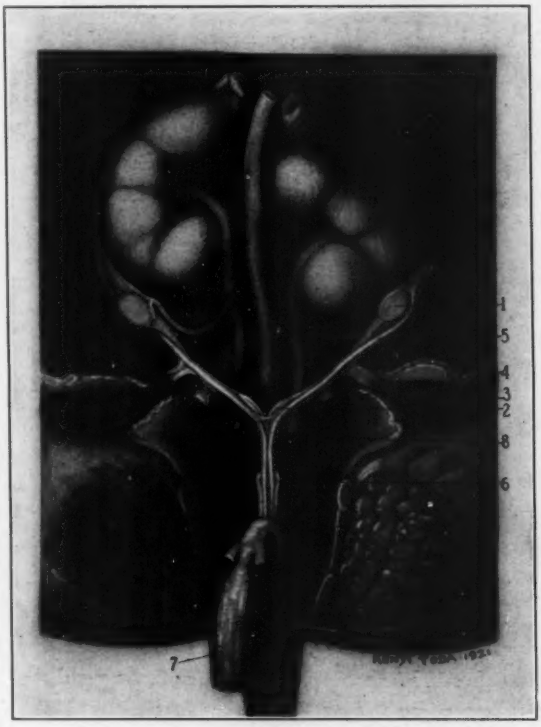

FIG. 4. Anatomy of the urinogenital system of the free-martin of Case 81, 14 cm. long. Described in the text. Greatest length about 14 cm. 1. Gonad; 2. Rudiment of cornua uteri; 3. Gubernaculum; 4. Cut end of umbilical artery; 5. Wolffian duct; 6. Seminal vesicles; 7. Allantoic stalk reflected; 8. Ureter. (× 2.)

action of the male sex hormones requires qualification or sharper definition. The case we are considering would seem to have afforded the maximum opportunity in these respects, without the maximum result following. It should, however, be borne in mind that no one has yet investigated the stages of the free-martin from

32 cm. length to birth (about 100 cm.), and that the fœtal conditions have not, therefore, been adequately correlated with adult

FIG. 5. Anatomy of the urinogenital system of the free-martin of case 94, 19.25 cm. long. Described in text. Greatest length about 19 cm. 1. Gonad; 2. Wolffian duct; 3. Gubernaculum; 4. Cut end of umbilical artery; 5. Wolffian duct; 6. Seminal vesicles; 7. Allantoic stalk reflected; 8. Ureter. (× 2.)

conditions. It would seem that the major portion of the growth of the gonad of the free-martin occurs in these missing stages, and

it is possible that in the present case the gonad would have grown considerably. But it could not have passed out of the body cavity, for the gubernacula did not enter the body wall in this case. And it is impossible that a male type of external organs could have developed in this case, for the definitive female type was already formed.

Cases at the other extreme from the present one also throw some doubt on the merely quantitative explanation of the degree of intersexuality. I refer to cases where the vascular interconnection is very delicate. One case of this kind, not previously reported, is especially striking (No. 81). In this case I at first doubted the existence of an actual arterial anastomosis, for the injection was not very successful. A careful examination, however, showed an exceedingly delicate artery from the male side passing far into the territory of the female chorion and there apparently anastomosing with an artery from the female. The reproductive system of the free-martin in this case (stage of 14 cm.) was typically intersexual (Fig. 4) and similar in all essential respects to the case shown in Fig. 15 of my former paper: gonads reduced, ducts of the male type with rudiments of the cornua uteri persisting, however, gubernacula present, etc. Indeed, this case is certainly more modified than No. 78.

Keller and Tandler (1916) describe a case in which the vascular arrangements found seemed inadequate to account for the typically deformed condition of the reproductive organs of the free-martin. In this case they state that no strong vascular anastomoses were demonstrable, nevertheless the female exhibited the typical malformation of the reproductive system. They add that a welt-like connection similar to that which usually carries the connecting vessels was found between the main vascular trunks on the two sides, but that it possessed no lumen. They consider this as pointing toward the possibility of an earlier typical connection that became interrupted secondarily.

All free-martins that have been described depart very widely from the female anatomical plan, and also very widely from that of the male. If we were to imagine a sex scale of, say, twenty points, in which points 1, 2, and 3 on the left include the normal female range and 18, 19, and 20 on the right the normal male

range, then I would say, as a matter of quite arbitrary judgment, that the free-martins would occupy points 8 to 13, inclusive, possibly encroaching in some doubtful cases on points 14 and 15. The free-martins, in other words, form an exceedingly well-defined group without intergrades to normal female or normal male. Within the group of free-martins there is considerable range of variation which may be classified according to degree of approximation toward the male type, as B. H. Willier (1920) has shown for the gonads. The ducts also would permit of a similar classification, though it is not certain that the classification by gonads and ducts would always agree.

The absence of intergrades to the female side, and the absence of correlation between the size of the vascular anastomosis and the degree of intersexuality, indicates an "all-or-none" type of reaction in formation of the free-martin; presumably, therefore, the differentiating action concerns a rather definitely delimited period of development and does not require a maximum amount of the male hormones. If this be so, variations of the quantity of the hormones within wide limits would not be significant for the typical reaction. But variation in time of onset with reference to the preceding degree of differentiation of the ovary and other organs of the female may be sufficient to explain in part the range within the free-martin series (cf. Willier, 1920). The matter is discussed further in section 6 (p. 70).

It is interesting to note that Pézard (1920) concludes that the morphogenetic effects of transplanted testicular fragments on castrated cocks also follows an "all-or-none" law; "as soon as the functional threshold is passed, whatever may be the mass of the active gland, a cock takes on as a whole its secondary sexual characteristics. It appears that these manifestations can not be fractionated."

5. On the Occurrence of Seminal Vesicles in the Fœtal Free-martin.

In my study of the anatomy of the fœtal free-martins (1917) the existence of seminal vesicles in the stages in question was doubted, although they appear in the males in much earlier stages. On the other hand, seminal vesicles appear usually to be present

after birth in free-martins, and I therefore assumed that their appearance in the free-martin was probably belated. A reëxamination of the material, however, shows that they are always present in fœtal free-martins in which the lower part of the Wolffian ducts is developed. Fig. 5 from a specimen hitherto not recorded (case No. 94) shows the typical condition in a free-martin with well-developed Wolffian ducts.

Reëxamination of the specimens figured in my 1917 paper shows similar structures, sometimes more slender, in those represented by Figs. 14, 15, 17, 18, 20, 21, 22, 24, 25, and 27. On the other hand, the specimen shown in Fig. 26 had no Wolffian ducts and no seminal vesicles. The other specimens figured were not available for reëxamination. The specimens were drawn correctly, as dissected at the time the drawings were made; it required further dissection to reveal the seminal vesicles (cf. Fig. 5 of this paper with the figures of the 1917 paper).

This correction removes the necessity for the special assumption made in the 1917 paper that these organs develop out of their due time.

6. The Earliest Stage of the Free-martin.

The smallest free-martin hitherto described is 7.5 cm. greatest length, case 19 of my 1917 paper. The gross anatomy was described and figured by myself, and the histology by Miss Chapin (1917). The normal female characters are highly modified at this stage; the ovary exhibits a serous covering and albuginea similar to that of the male, absence of the ovarian cortex, and presence of the medullary components of the ovary. In the normal ovary of the same stage the cortex is highly developed, though Miss Chapin states that the cords of Pflüger are not yet separated from the germinal epithelium. The Müllerian ducts of the free-martin have begun to degenerate similar to a normal male of corresponding age (Chapin, p. 459). It is, therefore, apparent that the modifications of the free-martin must start at a considerably earlier stage.

From a study of the chorionic vesicles of normal single pregnancies I concluded that the opportunity for fusion of embryonic membranes from a two-sided twin pregnancy is present from the

10-mm. stage of the embryos, and for vascular anastomosis from the 19-mm. stage, or slightly earlier, because the allantois passes completely through the body of the uterus at that time. This antedates the beginning of visible sex differentiation which I estimated then at about 25 mm., confirmed since by a much more extensive study.

These considerations indicated the possibility of exchange of blood between twin embryos before the beginning of sex differentiation, but it was not demonstrated that this actually occurred, nor yet how early the blood of the male had any sex hormone contained in it.

The case about to be described (No. 62) gives a fairly definitive answer to these problems. The point of chief interest, demonstrated below, is that the hormones in the male blood have produced a practical inversion of the ovary of the female twin at a stage of 3.75 cm., and must hence already have been active for some time. The weight of a male embryo of 3.8 cm. is 3.65 gr.; the weight of its two gonads is 0.0025 gr., or about one fifteen-hundredth that of the embryo. The quantity of interstitial tissue can be only a small fraction of the total mass of the gonads. Nevertheless the secretion of this relatively minute fraction of tissue diluted by the total blood of both embryos is sufficient during growth of about one centimeter in length to reverse the normal course of development of the ovary of the female. I know of nothing that gives a more profound impression of the chemical aspect of development than this demonstrable fact.

Case No. 62: ♂ 4 cm. greatest length, ♀ 3.75 cm.; one in each horn of the uterus; collected in February, 1917. Only one of the maternal ovaries was present and it contained a single corpus luteum. The two chorions were fused so completely that the actual place of fusion can be determined only approximately. Injections of the umbilical arteries with a starch mass did not pass from one side to the other, but at least one perfectly distinct, though very fine, arterial anastomosis by side branches of the main arteries of the two sides could be demonstrated under the binocular.

The two embryos were at first diagnosed doubtfully as males and hence received no further attention for a long time, until I had become familiar with definite external characteristics that en-

abled me to distinguish the sexes with certainty as early as 3 cm. greatest length. They were then readily diagnosed as male and female, respectively, in their external characters. They were carefully studied and compared with normals, with the result that the reproductive system of the female was found to be already definitely modified in the characteristics about to be described.

Fig. 6 shows the external organs of the free-martin. The short

FIG. 6. Free-martin 3.75 cm. greatest length; Case No. 62. × 6. 1. Umbilical cord; 2. Teats; 3. Phallus. 4. Homologue of scrotal sac of male, formed in all females; 5. Urinogenital aperture; 6. Anus.

length of the perineum and the development of the urinogenital aperture are distinctive female characteristics. In the male the perineum is several times longer and marked by a ridge; there is no urinogenital aperture in the male, and the phallus is placed more anteriorly with reference to the scrotal sacs, which are entirely similar in the two sexes.

Figs. 7, 8, and 9 show the internal urinogenital organs of the free-martin (Fig. 7), of its male twin (Fig. 8), and of a normal female 3.6 cm. greatest length (Fig. 9). These figures are drawn

with the utmost care as to sizes and proportions of the parts. The much smaller size of the gonads of the free-martin is apparent at a glance. The actual measurements giving the average of the two sides in each case are: free-martin — 1.727 mm. × 0.818 mm.;

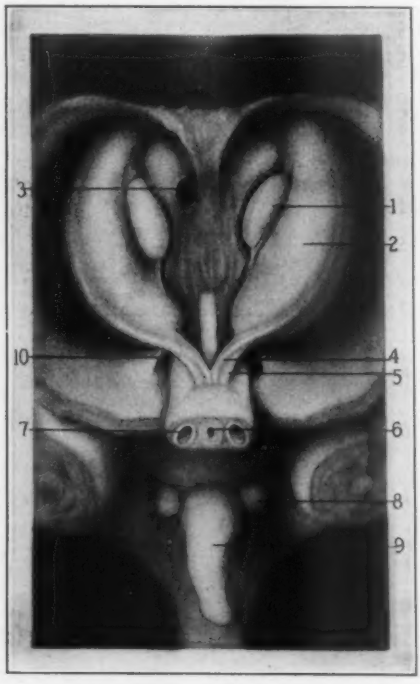

FIG. 7. Internal urinogenital organs of free-martin No. 62. 3.75 cm. greatest length, × 6. 1. Gonad; 2. Wolffian body; 3. Vena cava inferior; 4. Müllerian duct; 5. Wolffian duct; 6. Allantois; 7. Umbilical artery; 8. Homologue of scrotal sac of male; 9. Phallus; 10. Inguinal ligament ("round ligament" of uterus).

male twin — 2.622 mm. × 1.346 mm.; normal female — 2.568 mm. × 1.346 mm. The third dimension exhibits at least an equal difference, as may be seen by comparison of the outlines of cross-sections, Fig. 10, *a*, *b*, *c*, drawn at identical magnifications. The male and female normal controls may be regarded as typical for size of the gonads at this stage, for I have examined several other

specimens of each sex between 3 and 4 cm. in length for comparison. The hormone of the male has thus already operated so as to inhibit the growth of the gonad of the female to a very marked extent.

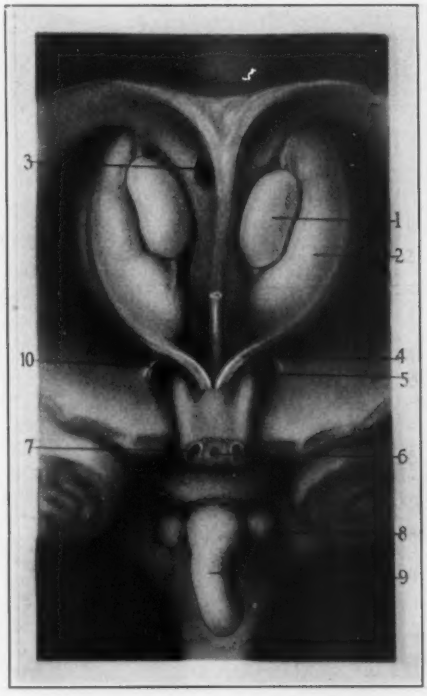

FIG. 8. Internal urinogenital organs of male twin No. 62. 4 cm. greatest length, × 6. 1. Gonad; 2. Wolffian body; 3. Vena cava inferior; 4. Müllerian duct; 5. Wolffian duct; 6. Allantois; 7. Umbilical artery; 8. Scrotal sac; 9. Phallus; 10. Inguinal ligament (part of gubernaculum).

The inhibition in growth is accompanied also by inhibition of the characteristic differentiation of the ovary, though apparently not as yet, to any marked degree, by stimulation of the male homologues in the ovary. In Figs. 11, 12, and 13 comparison is made between the histological structure of segments of those parts of the section outlines of Fig. 10 indicated by the reference num-

bers. In the male (Fig. 12) the germinal epithelium is already reduced to a single layer of nuclei, the albuginea has undergone

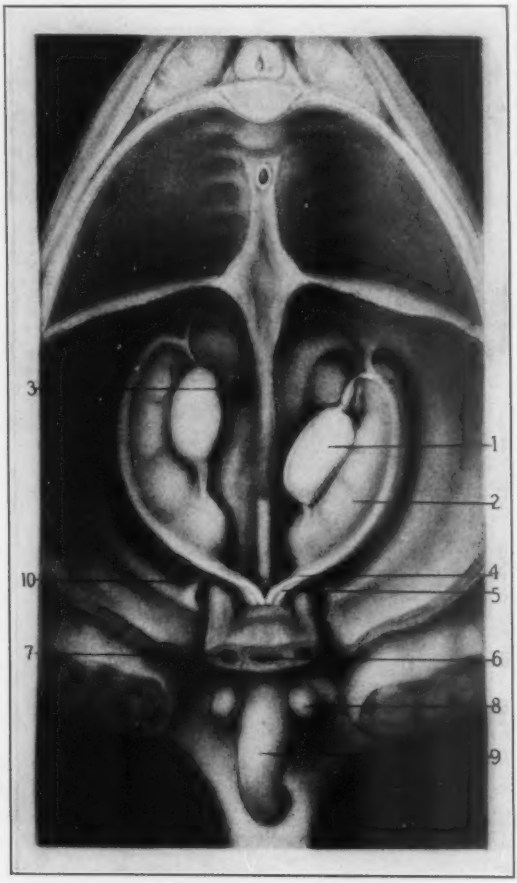

FIG. 9. Normal female, 3.6 mm. greatest length (No. 54c) × 6. 1. Gonad; 2. Wolffian body; 3. Vena cava inferior; 4. Müllerian duct; 5. Wolffian duct; 6. Allantois; 7. Umbilical artery; 8. Homologue of scrotal sac of male; 9. Phallus; 10. Inguinal ligament.

definite fibrous differentiation, and the primary sex cords are forming definite tubule rudiments with abundant interstitial tissue be-

tween. In the female (Fig. 13) the germinal epithelium (about 140 μ in thickness) is in very active proliferation forming several, to many, layers of nuclei. Cords of Pflüger are definitely indicated in the new cortex thus established. The primary albuginea contrasts with that of the male by its more embryonic type. The primary sex cords are not sharply differentiated from the stroma

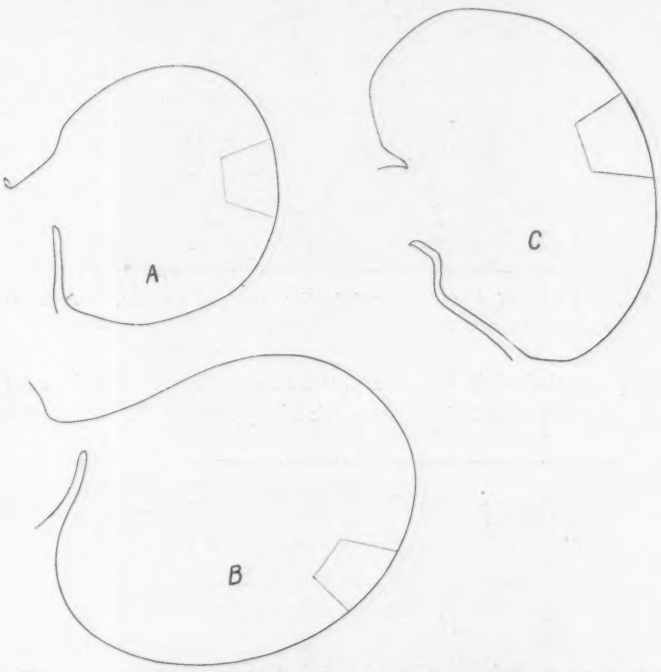

FIG. 10. Outlines of cross sections through center of the gonad, (*a*) of free-martin No. 62, (*b*) of male twin No. 62, (*c*) of normal ♀ No. 54*C* illustrated in Fig. 9.

of the medulla. The free-martin (Fig. 11) shows the same three layers. The primary albuginea and the medulla do not differ much from those of the female, but the preservation in formalin was not good enough for detailed comparisons on this point. The germinal epithelium is thicker than in the male, but only about one fifth on the average of the thickness of the female. It is, indeed, not as thick as that of a normal female of 3.0 cm. and contrasts

also with it in the much sparser distribution of the nuclei, evidence of complete cessation of its activity.

It would thus appear that the characteristic proliferation of the

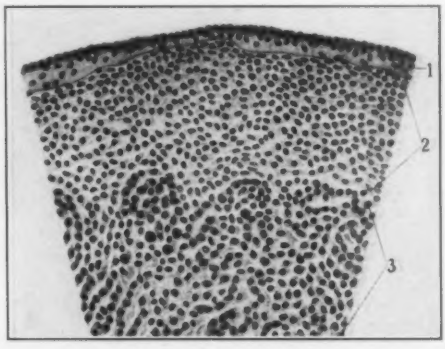

FIG. 11. Detail of area designated in Fig. 10*a*. Free-martin, No. 62. 1. Germinal epithelium; 2. Albuginea; 3. Primary sex-cords (medulla).

ovary was halted in the free-martin at a stage not later than 3.0 cm. As will appear from the work of my student, K. F. Bascom,

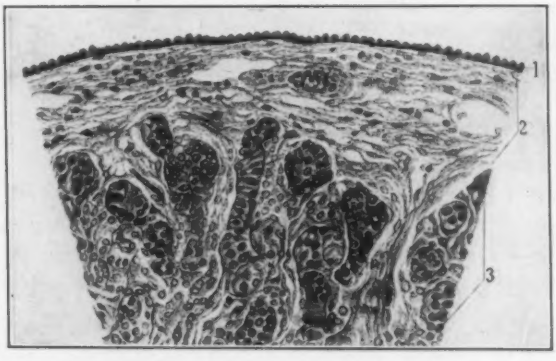

FIG. 12. Detail of area designated in Fig. 10*b*. Male twin, No. 62. 1. Germinal epithelium; 2. Albuginea; 3. Primary sex cords (seminiferous tubules) and interstitial tissues.

soon to be published, recognizable differences between the testis and ovary, concerning the germinal epithelium and albuginea, may

be traced back to the stage of 25 mm. at least. Comparison of these leads to the conviction that in this free-martin ovarian differentiation had begun, and that modification did not date from a stage in which the prospective differentiation of the gonad, whether as ovary or testis, is still indistinguishable. However, the work of Chapin (1917) and of Willier (1920) has shown that in later fœtal and post-natal stages nothing remains comparable to the cortex of the ovary.

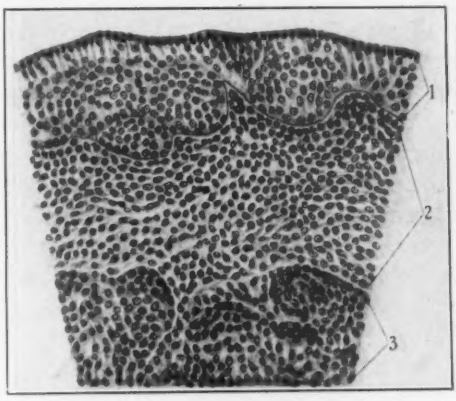

FIG. 13. Detail of area designated in Fig. 10c. Normal female No. 54c. 1. Germinal epithelium (cords of Pflüger); 2. Albuginea (primary); 3 Primary sex-cords (medulla).

The ostium tubæ abdominale of the normal female is conspicuous at the anterior end of the mesonephros (Fig. 9). It lies in the same position in the normal male (Fig. 8), though it is less conspicuous. In the free-martin (Fig. 7), on the other hand, it can not be seen in the dissection, and was found in sections a considerable distance from the anterior end of the mesonephros, as though the Müllerian duct had failed to grow in length after its formation, and thus its anterior end had become displaced. For the rest there is practically no difference in the ducts in the two sexes; and naturally none was to be expected, nor was any found, in the free-martin.

The feature of most general interest in this case is the demonstration that a specific sex hormone is being produced by the male,

presumable from its testis, at a period very closely approximated to the beginning of visible sex differentiation, which has a specific inhibitory action on proliferation of the germinal epithelium and on the rate of growth of the ovary as a whole.

This case is an isolated one so far as the early stage is concerned. There are, therefore, certain restrictions in generalizing from it. The other evidence that we have demonstrates, however, that the general relations in this case are typical. But it is probable that actual vascular connection between the two embryos may, in some cases, be established earlier, and, in other cases, somewhat later. We considered in section 4 the idea that the quantitative aspect of the vascular interchange is probably not an important factor in the degree of intersexuality of the free-martin; that we are dealing, in other words, with a reaction of the "all-or-none" type. So far as we can see, then, the principal factor, so far as hormones are concerned, in determining the range of variation within the free-martin series is probably the time, in relation to the early stages of sex differentiation, at which vascular interchange is established. But to supplement this, I believe it is necessary to assume that different individuals vary in the state of balance of the zygotic sex factors, and this may influence the quantitative effect of the hormone factor.

It is probable from these results that the ovary does not produce a sex hormone at such early stages. If it were doing so, some reciprocal effect upon the male ought to be observed in some cases at least. The histological evidence is just as negative as the physiological in the case of the ovary, while in the case of the testis fully differentiated interstitial cells are found from the 3 cm. stage of the embryo on (Lillie and Bascom, '22). Mr. Bascom will deal with this subject in detail in his study, but I may here call attention to the fact that all students of ovarian hormones locate the cells of origin in the cortex of the ovary, and that this part of the ovary is in its earliest state of origin in the stages in question, and exhibits as yet no differentiation of cells of internal secretion.

Moore (1921) and Sand (1919) have denied Steinach's idea of a mutual antagonism of testis and ovary of mammals by showing that the coexistence of both gonads in the same body is possible. The present results show, however, that prior to the forma-

tion of the ovarian hormone the testicular hormone is antagonistic to growth of the ovary. Presumably, therefore, the normal growth of an ovary in the presence of a testis, as in the experiments of Moore and Sand, depends upon a protective action that the elaborated ovarian hormone exerts against the testicular hormone. I do not think that Moore (1921, p. 168) is entirely correct in his opinion that hormone action is never characterized by inhibition, and that in the case of the free-martin the transformation of the gonad is due solely to the stimulating action of the male hormone. The gonad of the free-martin is unquestionably inhibited not only in its total growth, but also in its histogenesis by the male hormone; later yet the growth of the Müllerian duct is halted and degeneration also sets in owing to the presence of the male hormone. It is not until a still later period that the stimulating effects of the male hormone on the sex cords and rete of the gonad and on the epididymis and Wolffian duct of the free-martin become apparent. There is certainly an element of truth in the contention of Steinach, Lipschütz, and others that the sex hormones inhibit heterologous sex characters in addition to stimulating the homologous ones.

The great and important advances made in recent years in the study of sex hormones have led certain authors to extreme positions concerning their economy in the organism. Lipschütz (1919, p. 390 ff.) has given the boldest statement in his theory that the embryonic soma is primarily asexual, and that sex characters are secondarily imposed upon it by the differentiation of a male or female "puberty gland." In the discussion of this statement we should no doubt run foul of definitions. Whether it is to be understood under the presupposition of the zygotic *determination* of sex or not is not clear. If so, it confines itself to the phenomena of sex *differentiation*. The primary differentiation, therefore, which itself requires explanation, would be presumably that of the misnamed "puberty gland." This is, however, demonstrably not the case (Lillie and Bascom, '22). Moreover, if there were no other factor at work in determining the sex differentiation of embryonic primordia than the specific sex hormone, it is difficult to understand why the free-martin, which receives only male sex hormones, should not become completely male. It is obvious that the male hormone

is acting against resistance in the female soma; moreover, this resistance is not that of already differentiated parts, for the hormone is introduced before sexual differentiation; it is rather a constitutional resistance native to the determined sex. The phenomena can be understood only on the assumption that the zygotic sex-determining factors are also sex-differentiating factors in mammals as in insects. These factors are reinforced very early by hormone production in the male of mammals, but relatively very late in the female. Such reinforcement can be thought of in the sense of an autocatalytic reaction (as suggested by certain writers, cf. Goldschmidt, R., Huxley, J., 1920), in which the hormones are regarded as by-products of the sex-determining factors present in the zygote, that accelerate the progress of the differentiation.

General Discussion.

On account of certain misunderstandings that appear in the literature since publication of my 1917 paper, I would like to point out the limitations of the theory of the free-martin. I have so far confined the explanation to the bovine free-martin, though indicating the possibility that it may have a wider application. Minoura's experiments (1921) confirm the general applicability of the principle. The explanation is not supposed, however, to apply to all cases of hermaphroditism in cattle; it is not only possible, but probable, that certain cases rest upon a zygotic basis, for hermaphroditism is reported for single births in some cases. The possibility, therefore, exists of an accidental combination of such a case with twinning, though it would necessarily be very rare. There is, however, a sufficient basis for receiving with a certain amount of skepticism cases alleged to be due to twinning in which the history is based on statements of farmers, and not on the personal observations of the investigator. Most of the cases in the literature are, as a matter of fact, of this nature. There is no good reason for rejecting them so long as they are conformable to cases known to be due to twinning; but when they are quite unconformable, especially if they involve extensive modifications of the external parts, judgment may be reserved until proof is forthcoming that such modifications may be due to twinning.

I regard the explanation of the bovine free-martin which I have

given as proved by the large body of evidence accumulated by Keller and Tandler and by myself. Esther Rickards and F. Wood Jones (1918) find unconformable cases in goats described by themselves and others and conclude that these "show the unsoundness of Lillie's theories": A pair of apparently female goat kids turn out to be quite identically predominantly male in their anatomy; "it is affirmed of a celebrated he-goat 'that one season *every* kid he sired, eleven in number, was a hermaphrodite'" (quoted from Davies, 1913). Such cases imply a genetic foundation which certainly deserves study and promises interesting results, but they obviously do not have any bearing at all on the bovine free-martin.

Keller and Tandler (1916) investigated the membranes in four cases of triplets and two cases of twins in goats. In two of the triplet cases three corpora lutea were found in each case, and in one of the twin cases there were two corpora lutea. The ovaries were not available in the other two cases. The chorions were fused, but the individual fœtal circulations were separate, as in sheep (cf. Lillie, 1917). The fœtuses of four of these cases, which included both sexes, were examined and found normal in the anatomy of their reproductive organs. These findings thus confirm, as far as they go, the idea that hermaphrodites in goats are not usually a result of twinning as such; but they do not exclude the possibility of the occasional occurrence of intersexual conditions due to hormones. It is obvious that goats are not favorable material for the investigation of the action of sex hormones in fœtal life.

Hartman (1920) suggests that certain cases of hermaphroditism in mammals may be interpreted as "reciprocals" of the free-martin—that is, cases in which the male of a two-sexed pair is modified in its sexual development by hormones of the female. The evidence that he offers is of the slenderest description. Moreover, if, as seems probable, the female in mammals does not produce sex hormones during the early part of fœtal life, the possibility of "reciprocal" free-martins would seem to be excluded. The source of the ovarian sex hormone is by no means so certainly ascertained as that of the testicular hormone. The weight of opinion inclines to the view that the cells of origin in the ovary are

derived from atretic follicles. If this view should be confirmed, the time of first formation of specific female sex hormones would be postponed in most species until after birth.

Doncaster's suggestion (1920) that the tortoise-shell tomcat may be a female transformed by hormones of a male partner in fœtal life has been dealt with by C. C. Little (1920, 1921), who finds no vascular connections between the membranes of fœtal cats. The suggestion, however, leads me to emphasize the finding that conditions in the free-martin, which offer probably the most favorable possible opportunity, do not lend themselves to the idea that complete sex inversion by hormones is a possible thing. I am led to emphasize this because Julian Huxley (1922, pp. 201 and 214) also states, basing his statement on the free-martin, that hormones may produce all stages of conversion of one sex into the other. Although I have paid particular attention to this point, I have been able to find no evidence for it. On the contrary, the conversion of the female sex in the case of the free-martin stops very far short of complete inversion. There is a wide gap between the most extreme case and the normal male, and the statistics do not favor the suggestion that in some cases this gap may be crossed at a bound, leaving no intermediates (cf. p. 50). I believe, however, that proper caution admonishes us to realize that the limits of hormone action may vary, even widely, in different species. Both Steinach and Moore, for instance, find considerable differences between the rat and guinea-pig in the feminizing effect of ovarian grafts on castrated infantile males. I do not, therefore, mean to deny the possibility of complete sex inversion by hormones for all species, but merely to emphasize that the case is not proved for any species.

So far it has not been shown that the explanation of the intersexual condition in the free-martin is of wider application for the explanation of intersexuality in mammals. The theories must, however, develop along two main lines, viz.: either that of unbalanced sex factors or of unbalanced hormones. The former is a problem in genetics, which will be advanced by actual breeding experiments, for which goats seem to offer unusual advantages owing to the relative abundance of hermaphrodites in certain breeds, and the negative evidence against the theory of unbalanced

sex hormones in this case. The sex hormones may be unbalanced as against each other or as against genetic sex factors in other ways than by anastomosis of fœtal circulations in different-sexed individuals. As I pointed out previously, there may be a breaking down in the presumable defence mechanism of the placenta that protects the embryos from the sex hormones of the mother. Possibly other causes may also exist which in the present state of development of sex hormone theory can not be anticipated.

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Appendix.

Supplementary List of Cattle Twins Examined in Utero.

The list published previously included the first 57 cases. A comparable table of cases 58–92 is herewith given to make the record complete.

SUPPLEMENTARY LIST OF CATTLE TWINS EXAMINED IN UTERO.

No.	Sex. ♂	♀	☿	Size.	Chorion.	Maternal Ovaries.
58	2			14.5 cm. each.	Single.	Ovaries absent.
59		2		19.5 cm. each.	Single.	Ovaries absent.
60	1		1	♂ 16 cm., ☿ 15.2 cm.	Single.	One ovary present with corpus luteum.
61		2		23 cm., 24 cm.	Single.	Ovaries absent.
62	1		1	♂ 4 cm., ☿ 3.75cm.	Single.	One ovary present with corpus luteum.
63	1	1		22 cm. each.	Chorions separate.	Ovaries absent.
64	1	1		♂ 14 cm., ♀ 13.75 cm.	Chorions separate.	Both ovaries present. Corpus lut. in each.
65	1		1	♂ 17 cm., ☿ 16.75 cm.	Single.	Both ovaries present. Corpus lut. in each.
66	See Note 1.					
67		2		10 cm., 10.25 cm.	Single.	Ovaries absent.
68	2			9.5 cm., 10.3 cm.	Single.	Ovaries absent.
69	2			7.8 cm., 8.2 cm.	Single.	Both ovaries present. Corpus lut. in each.
70	2			13 cm. each.	Single.	One ovary present with corpus luteum.
71	2			17 cm., 18 cm.	Single. Narrow connection.	Both ovaries present. Corpus lut. in each.
72		2		28.5 cm. each.	Single. Both embryos in one horn of uterus.	Both ovaries present. 2 c. l. in one. None in other.
73	2			6 cm. each.	Single.	Both ovaries present. Corpus lut. in each.
74		2		12.5 cm., 13.5 cm.	Single.	Ovaries absent.
75	1		1	16 cm. each.	Single.	Ovaries absent.
76		2		15.5 cm., 16 cm.	Single.	Both ovaries present. Corpus lut. in each.
77	1		1	♂ 10 cm., ☿ 9.4 cm.	Single.	Ovaries absent.
78	1		1	32 cm. each.	Single. Both embryos in one horn of uterus.	Both ovaries present. Two corpora lutea in one. None in other.

Note 1.—Five days' old free-martin, twin to bull. Preserved material for histology. See Willier ('20).

No.	Sex. ♂	Sex. ♀	Sex. ☿	Size.	Chorion.	Maternal Ovaries.
79		2		26 cm., 27 cm.	Single.	One ovary present with corpus luteum.
80		2		16 cm. each.	Single. Both embryos in one horn of uterus.	Both ovaries present, but only one corpus luteum.
81	1		1	14 cm. each.	Single. Narrow connection.	Ovaries absent.
82	2			13 cm. each.	Single.	One ovary present with corpus luteum.
83		2		12 cm. each.	Single.	Both ovaries present. Corpus lut. in each.
84		2		15 cm. each.	Single.	Both ovaries present. Corpus lut. in each.
85	1		1	11.5 cm. each.	Single.	One ovary present with corpus luteum.
86		2		Note 2.		
87		2				
88		2				
89	2					
90	2					
91	1		1	15 cm. each.	Single.	Ovaries absent.
92	1		1	♂ 12 cm., ☿ 11.5 cm.	Single.	Ovaries absent.
93	1	1		♂ 10.25 cm., ♀ 7.75 cm.	Narrow non-vascular connection.	Both ovaries present. Corpus lut. in each.
94	1		1	♂ 19.25 cm., ☿ 19 (?) cm.	Single.	Both ovaries present. Corpus lut. in each.
95	2			4 and 5 cm.	Single without constriction. Both embryos in one horn.	Both ovaries present, two corpora lutea in one; none in the other.
96		2		Not recorded.		Absent.
97	1		1	About 15 cm. each.	Single, without constriction. Both embryos in one horn.	Absent.

Note 2.—Numbers 86 to 90 inclusive were collected during my absence in January and February, 1920; no records but sex were taken

COPPER, ENZYMES, AND FERTILIZATION.

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I. INTRODUCTION.

The inhibition of development in *Arbacia* by infinitesimal quantities of copper salts has lately been employed by F. R. Lillie (21[1, 2]) as an expedient in studying the fertilization reaction. The results are highly suggestive and the novelty of the procedure altogether likely to inspire much further work. Yet even now there are complications to be dealt with before one can realize on the possibilities which apparently inhere in the method.

In the first place, there is the matter of effective concentration. This has two aspects: how much of the copper salt added to sea-water actually remains in solution and what proportion of the permanently soluble fraction reaches the eggs? Moreover, is the chorion involved? There is no reason for thinking that copper would escape concentration in the egg-jelly since, as I have recently shown (22[1]) this is true of all the salts to which the egg is normally exposed. And, finally, is it possible for a marine organism to carry a "copper-avid substance in the cortex of the egg" and escape the all but inevitable consequence?

II. THE PRECIPITATION OF COPPER SULPHATE IN SEA-WATER.

The first step was a study of the solubility of copper sulphate in sea-water. Since my program involved reliance on chemical, rather than physiological, means of detection, I employed concentrations well within the sensitivity range of my first methods. I therefore prepared a standard solution of $CuSO_4 . 5H_2O$ in distilled water such that each cubic centimeter corresponded to one milligram of the original salt. This was equivalent to .276 mg. of metallic copper per c.c., or Cu $= m/230$ and $n/460$.

This standard was compared colorimetrically with a duplicate solution in sea-water. After correcting for the few drops of ammonia added in order to intensify the blue color, it was found

in three closely concordant experiments that 36.9 per cent. of the copper had been precipitated in the sea-water—in all probability—as insoluble basic carbonates. The sea-water, therefore, retained in solution a trifle over .174 mg. Cu per c.c.—the equivalent of Cu = $m/365$ or $n/730$.

III. The Removal of Copper from Solution by Normal Eggs.

The precipitated fraction of the copper sulphate interferes seriously with attempts to measure colorimetrically the quantities actually removed by the eggs. Not only is the turbidity of the solution objectionable, but the precipitate adheres mechanically to the chorion, which in turn goes slowly into solution. It is absolutely essential to eliminate the copper carbonates.

However, a concentration of $n/730$, even after the carbonates are removed, often destroys the eggs in all except brief exposures. This difficulty can be overcome by dilution to $n/1{,}460$. At this concentration .2 c.c. of dry ripe eggs in 75 minutes reduced the copper content of 14.8 c.c. of solution as follows:

Lot *A* from $n/1{,}460$ to $n/1{,}810.4$.
Lot *B* from $n/1{,}460$ to $n/1{,}766.6$.
Lot *C* from $n/1{,}460$ to $n/1{,}785.5$.

Very definitely, then, *Arbacia* eggs are able to remove copper from solution, yet, despite their close agreement, the figures in reality have no quantitative standing. They completely mask an important source of error.

IV. The Rôle of the Chorion.

If the chorion takes part in lowering the concentration of copper solutions, eggs without jelly should lose at least part of their effectiveness. But to demonstrate this is not easy. The very removal of the chorion, of itself, introduces variables which, in the present case at least, might be increased in number or aggravated by the use of dilute hydrochloric acid as a solvent of the jelly. This restricts us to the mechanical methods with their relative violence and uncertainty. One can never feel confident that every egg has lost its chorion, nor that the shaking, flipping—or whatever one does—leaves every egg intact. Some control of these errors

is possible by microscopic examination in suspensions of india ink, and, as a matter of fact, I never employed material unless the eggs appeared uninjured, nor unless at least 90 per cent. failed to reveal the jelly. However, it was impossible to offset the fact that equal volumes of normal and dechorionized eggs can not contain equal numbers. It is not surprising, therefore, that comparisons with normal material should prove variable; nevertheless, in view of my other experiments, I did not expect to encounter the irregularities actually found. These were so great that the results are best appreciated in the following form:

	Excess Copper Removed in 75 minutes from 10 cc. M/1460 Solution by .2 cc. Normal Eggs		.2 cc. Dechorionized Eggs
Lot A	23.3%		
Lot B			11.5%
Lot C	29.5%		
Lot D	40%		
Lot E			2.0%
Lot F			8.3%
Lot G	13.7%		
Lot H	54.0%		
Ratio	5.7	:	1

This clearly does not indicate that dechorionized eggs remove no copper from solution, but that normal eggs, on the average, remove more. If we neglect the numerical differences in equal volumes of the two classes of eggs—a handicap distinctly against the normal material—we can say that the copper profit of normal was to that of dechorionized eggs at least as 5.7 : 1.

It follows that the chorion is heavily involved in the removal of copper from solution. Yet the amounts taken up can not be determined even from my original values; nor can these be used as a basis for predicting the precise outcome of any particular competition for copper between normal and dechorionized eggs. Indeed, the results, though valid enough, once more mask a fundamental source of error.

V. Irregularities in the Removal of Copper from Solution.

The irregularities encountered were of several sorts. Equal quantities of eggs, during equal exposures, did not invariably ap-

pear to remove equal quantities of copper from a $n/1{,}460$ solution. In fact, in certain instances, the solutions after exposure to the eggs seemed to contain more copper than I had added. With dechorionized material the results were almost bizarre. Such eggs usually appeared to absorb less than the normal, though at times the opposite was true. Their most frequent irregularity was the apparent addition of copper to the sea-water. Where was the error?

I sharpened my technique as much as possible. After each experiment a few drops of sulphuric acid were added to the solution to be tested in order that any copper carbonates formed during the experiments might be converted. I also used ammonia to intensify the colors. Nevertheless the harvest of inconsistencies continued.

It was then that I recalled Lillie's "copper-avid substance in the cortex of the egg." This might explain everything, yet only if it could be shown that the untreated normal *Arbacia* egg actually contains copper.

VI. Demonstration of Copper in *Arbacia* Eggs.

A. Preliminary Considerations.

Was there any likelihood of demonstrating copper in the normal egg? In the literature I found the following especially pertinent facts:

100 gr. fresh *Asterias rubens* contain 2.45 mg. Cu.

100 gr. fresh *Stichopus regalis* contain 2.83 mg. Cu.

100 Sepia eggs contain 0.5–0.8 mg. Cu.

100 gr. dry hen's yolk contain 2.00 mg. Cu.

The first three items are from Aron's ('09) discussion in Oppenheimer's Handbuch; the last from an investigation by Fleurent and Levi ('10[1]).

Measurable quantities of copper, then, have been reported for certain echinoderms and at least two kinds of eggs.

B. Qualitative Tests for Copper.

It is hardly necessary to discuss the very large number of preliminary tests made at Woods Hole during the summer of 1921.

It is sufficient to say that whenever I was able to avoid interference, due to small amounts of iron, I found positive indications for traces of copper. Material was, therefore, prepared for the more careful analyses made this winter at Amherst.

Immature eggs were taken directly from the ovaries; mature eggs were shed spontaneously under conditions which I have described elsewhere (22[1]) and which precluded any contact whatever with sea-water, fresh water, dermal secretions, appendages, or detritus of any kind. The eggs were tested with sperm and found to be normal.

Two c.c. of mature eggs dissolved in concentrated nitric acid constitute sample *A*. This was subsequently diluted to 50 c.c. with water doubly distilled from glass. Fractions of this material were used either directly or, after evaporation to dryness and incineration in glazed china crucibles. The ash was redissolved in sulphuric acid, usually neutralized with ammonia, and the volume restored to that of the original fraction. In all these operations great care is essential. All reagents were tested repeatedly and found negative for copper.

1. *Copper as Cupric Hydroxide*—$Cu(OH)_2$.

   1 c.c. of *A* evaporated to dryness and incinerated. Ash dissolved in H_2SO_4. Excess NH_4OH should give the blue of cupric hydroxide. Result: positive but exceedingly faint.

2. *Copper as Cupric Cyanide*—$Cu(CN)_2$.

   To 5 c.c. of *A*, added concentrated KCN solution. Should give yellow precipitate of $Cu(CN)_2$. The precipitate should dissolve in H_2SO_4 and this solution should give test for $Cu(OH)_2$. Result: positive and distinct throughout.

3. *Copper as Cupric Ferrocyanide*—$Cu_2Fe(CN)_6$.

   Ash from 10 c.c. of *A* redissolved and neutralized as before. To avoid interference by iron, the reagent, a 1:35 solution of potassium ferrocyanide, $K_4Fe(CN)_6$ in distilled water, was carefully run over the surface of the test solution as in the ring reaction for proteins with nitric acid. In the present case also a ring is formed; this quickly thickens—the upper layers being composed of the blue ferrocyanide of iron; the lower of brown cupric ferrocyanide.

4. *Copper as Cupric Xanthate*—$Cu(C_3H_5S_2O)_2$.

   Redissolved ash from *A*, in the presence of potassium ethyl xanthate should give a yellow color due to the formation of copper xanthate

The test which is sensitive roughly to 1 part in 50 million, is not vitiated by the presence of sea-salts nor by small amounts of iron, lead, nickel, cobalt, zinc, or manganese. Results: Positive and very decided.

5. *Copper as Crystalline Cupric Sulphate*—$CuSO_4 \cdot 5H_2O$.

After precipitation of the iron as hydroxide the ash was redissolved in sulphuric acid and drops of the solution allowed to evaporate to dryness on a slide. Under the microscope long blue needles corresponding to copper sulphate. The blue color greatly intensified by ammonia.

6. *Copper as Metallic Copper*—Cu.

5 c.c. of *A* plus excess NH_4OH. Copper if present in sufficient quantity should be deposited on tin-foil. Result: positive but faint. With aluminum, however, despite incompleteness, the deposition was very marked; film, rose-colored by reflected light; bright and typically copper-colored when polished.

7. *Copper by Electrolytic Deposition.*

10 c.c. of *A*. Platinum Electrodes. Voltage 2.6; amperage 1/10. At this tension copper and only copper can be deposited on the kathode. After 24 hours, result positive and distinct. Could not be weighed. Redissolved deposit and got positive test with potassium ethyl xanthate.

On the basis of this evidence it was no longer possible to doubt the existence of copper in the normal *Arbacia* egg. The basic necessity for a secretion of copper compounds or copper-bearing substances is therefore given. If such materials are held up temporarily by the chorion, the differences between normal and dechorionized ova, noted in sections IV. and V., would be explained. But do the eggs really secrete copper in any form? We could perhaps answer this question if we could associate the metal with definite structures and substances in the egg.

VII. Localization of Copper in the *Arbacia* Egg.

The problem calls for the localization of the copper. I employed direct methods as well as indirect. The eggs used were shed under the conditions previously mentioned and fixed in absolute alcohol. I studied both whole mounts and sections, 5 μ in thickness. The latter, after removal of the paraffin, like the unsectioned eggs, were treated with various reagents and examined in the opaque condition, wet or dry; or after clearing, as transparent objects, in glycerine or balsam. All the reagents were tested and again found negative for copper.

1. *Copper in the Egg Pigment.*

Since copper is an integral part of so many organic coloring matters, it was natural to examine first of all the pigment of the egg. This material, as is well known, is widely distributed in granular form in the cytoplasm. As the granules are most numerous in tangential sections, the pigment bodies are evidently concentrated near the surface. This also seems to be true, to some extent, immediately about the nucleus.

It is entirely justifiable to identify these granules as the source of the coloring matter secreted by the eggs. I consequently precipitated the pigment from normal egg secretion by the chloroform method described in an earlier paper (21[3]). After incineration an alkaline solution of the ash in twenty minutes gave a relatively heavy, though incomplete, deposition of metallic copper on aluminum. The deposit was dissolved and gave a deep yellow color on addition of potassium ethyl xanthate.

Direct proof of copper in the pigment bodies *in situ* is also possible. Tangential sections which contain more of these granules than others are the very ones that stain most heavily with hæmatoxylin. This we should expect in the presence of copper, though the test is not dependable if iron is also present. I therefore adopted a procedure employed in the microchemical analysis of minerals. The method depends on the formation of a triple nitrite of potassium, copper, and lead ('94).

To sections on the slide I added a trace of sodium acetate and a somewhat larger quantity of potassium nitrite. The whole was then acidulated with acetic acid. Last of all, a few grains of lead acetate. The presence of copper, as little as 0.05 microgram,[1] if crystals are desired, completes the conditions necessary for the formation of the triple salt, $K_2CuPb(NO_2)_6$, which is jet black.

In this test, which differentiates between iron and copper, the sections most darkened are the very kind most affected by hæmatoxylin. There is a marked graying of the cell contents to which a partial reduction of the pigment bodies contributes. The general effect is due to a decided increase in cytoplasmic granulation—the granules being excessively minute and, like the microsomes of

[1] 1 microgram (μgr.) = 1/1000th milligram.

the cytologists, black. If the material is kept moderately warm on a water bath and the reaction is permitted to continue for half an hour, the pigment bodies themselves become distinctly bluish or even black. This suggests a gradual unmasking of the copper in Macallum's sense ('12).

2. *Copper in the Egg Membrane.*

A definite visible membrane ('13[1]) invests the unfertilized *Arbacia* egg and constitutes a barrier through which pigment must pass in order to reach the outside. If now the pigment itself contains copper, the same thing might easily be true of the membrane through which it passes.

But the membrane is by nature yellowish-brown. This excludes tests depending on the formation of cupric cyanide, ferrocyanide, or xanthate. With hæmatoxylin a bluish tint develops, and this could be interpreted as evidence of copper if iron were absent. However, since the latter is almost certainly present, we must fall back on the triple-nitrite reaction.

In this the original native yellow-brown of the membrane is replaced with black. Under low powers the membranes are in very sharp relief. The oil immersion, however, resolves their uniform black, in optical section, into a series of irregularly spaced discontinuous beads connected by an exceedingly thin continuous black line.

This test was repeated on several sets of sections. The results were uniform and without inconsistencies. The evidence, then, that copper exists in the *Arbacia* egg membrane appears valid; yet the quantities involved are so small that I felt impelled to check the results on the hen's egg, whose vitelline membrane, I reasoned, should contain copper if the analysis of the yolk by Fleurent and Levi (*loc. cit.*) is correct.

This supposition was readily substantiated by four different methods.

The vitelline membranes were removed from the yolk and washed in a stream of running tap-water for twelve hours. In this way all but negligible quantities of yolk were removed.

After incineration a solution of the ash, upon the addition of ammonia, turned blue. This test was supported in neutral solu-

tion by a marked yellow brought about by potassium ethyl xanthate. Again the triple-nitrite test was positive. Under the microscope the treated membranes showed numerous irregular blotches and specks of black. By prolonging this treatment for thirty minutes and following it with a short exposure to 100° C., the membranes became closely speckled with black and underwent a discoloration quantitatively more in harmony with the very marked reaction gotten with ammonia and potassium ethyl xanthate. The copper in this membrane is, therefore, also "masked"; moreover, the discovery of copper in the *Arbacia* egg membrane does not stand alone.

3. *Copper in the Chorion.*

Since the pigment during its outward passage permeates the chorion, the latter necessarily contains traces of copper. There is also some direct evidence of this, for hæmatoxylin imparts to the jelly a light bluish tint, whereas the triple-nitrite test brings out a few scattered granules stained black. Infinitesimal quantities of copper, then, can be demonstrated directly in the chorion.

4. *Copper and the Cortex.*

Inasmuch as Lillie (*loc. cit.*) has assumed the presence of a copper-avid substance in the cortex of the egg, I paid especial attention to this region both in untreated sections and in those exposed to reagents for copper.

Unquestionably the cortex differs from the remainder of the egg. Yolk, pigment, and other visible granules are absent. The zone immediately beneath the egg membrane, even at very high magnifications, seems to be optically clear. In sections not exposed to reagents for copper the cortical layer appears blue—no doubt the result chiefly of refraction. Nevertheless the color is intensified by ammonia and hæmatoxylin and does not disappear entirely when viewed by light transmitted through ground glass nor after the sections have been cleared in glycerine, xylol, or balsam.[2]

Furthermore, perhaps on account of distortions through shrinkage, the blue layer, instead of being in immediate contact with the

[2] There are, of course, other effects of refraction throughout the egg.

membrane, not infrequently lies at a considerable distance beneath it. All this is indicative of a region differentiated from the remainder of the egg.

Is copper demonstrable in this layer? None of the direct methods could give an unequivocal answer, though the triple-nitrite test did reveal a few black granules similar to the black beads found in the membrane. This might be considered a demonstration of copper if the displacement of an occasional pigment granule in the process of sectioning could be altogether set aside. But there are other considerations.

The fertilization of egg fragments has been reported by numerous investigators, including myself ('13[1]), yet the correctness of these observations has been doubted because some egg fragments are incapable of fertilization. The difference is accounted for in the work of Chambers,[2] who finds that fragments derived exclusively from the interior of the egg can not be fertilized, whereas those containing a fair portion of the cortex respond like the original egg. This evidence, if not compelling, at least indicates more immediately than any other the physiological distinctiveness of the cortex and the necessity of cortical materials in fertilization.

Now, certain secretions of the egg are likewise necessary. This was first shown by F. R. Lillie ('13[2]), and subsequently by my sterilizations with charcoal ('21[4]). A logical combination of our two necessities traces the secretions to the cortex. But are they—or perhaps better, their forerunners—really there? For the normal egg this has not been demonstrated, nor is the cortex, in all probability, their only location; for egg fragments, with chorion and membrane both removed, the inference appears less hazardous. The concentration, then, of the secretions, or their forerunners, in the cortex of the egg may be assumed as a not unreasonable working hypothesis.

On the basis of isolable precipitates, Miss Woodward ('18) and I ('21[5]) have contended that these secretions contain at least two separate substances—a lipolytic ferment and a material which agglutinates spermatozoa. The immediate problem consequently narrows down to this: Is copper demonstrable in precipitated lipolysin and agglutinin?

[2] See Lillie's "Problems of Fertilization," p. 264.

A. Lipolysin—I.

The lipolysin first tested was prepared as follows: the eggs were permitted to secrete as usual; the agglutinin was salted out with ammonium sulphate, which incidentally also precipitates much of the pigment. After filtration, the lipolysin itealf was thrown down by means of barium, redissolved in dilute hydrochloric acid and precipitated by acetone which unless in great excess holds the remaining agglutinin in solution. The lipolysin so secured was a white powder free from pigment, though in the course of time it turned slightly purple.

After incineration a solution of the ash gave a positive reaction for copper with potassium ethyl xanthate.

Lipolysin—II.

This precipitate had exactly the same history as the foregoing except that charcoal was used in place of barium. Moreover, the powder did not change color but remained snow white and therefore may have been a somewhat purer product than lot I. By the xanthate test, lot II. also contained copper.

B. Agglutinin—I.

The agglutinin first examined came from *Asterias* egg-secretion which had been freed from lipolysin by the barium method. The xanthate test, carried out as before, gave a perceptible reaction for copper but as the separation from the other constituents of the secretion was incomplete, this result is inconclusive.

Agglutinin—II.

A second test was made, this time with *Arbacia* agglutinin which had been merely salted out with ammonium sulphate and contained therefore considerable quantities of pigment. In this case the xanthate test was markedly positive.

Agglutinin—III.

Arbacia agglutinin whose separation from pigment and other impurities was accomplished by chloroform, charcoal, and final differential precipitation from acetone. This material was negative for copper.

In all I examined five different lots of agglutinin, and in every case preparations whose purity I supposed to be high were the ones that proved to be either completely negative for copper, doubtful, or at most very slightly positive. It appears that copper is not an essential constituent or even regular associate of pure agglutinin.

These results, together with the triple-nitrite test and the considerations that suggest a cortical concentration of the secretions, justify my assumption that copper is present in the cortex of the egg immediately beneath the vitelline membrane. This copper,

however, is not associated with the egg pigment or the agglutinating material, but with the lipolysin, whose concentration in the cortex is thus one step nearer to being a demonstrated fact.

VIII. Copper and Enzymes.

Lipolysin is or contains a lipolytic ferment whose presence accelerates the hydrolysis of higher and lower fats ('22[3]) and the synthesis of ethyl-butyrate ('22[3]). The association of copper with this enzyme, though contrary to expectation, can not be evaded. Does the case stand alone?

Apparently not. For example, S. Yagi ('10[2]) finds a thirty-four fold increase in the copper content of the rabbit's liver when the animal is fed 4.8 gr. copper sulphate in assimilable doses during a period of seventeen days. This agrees perfectly with the results of Titze and Wedemann ('11) on the goat and those of Rose and Bodansky ('20[1]), who find the copper content of the oyster's hepato-pancreas to be twice that of the muscle. Although as yet no one has risked the suggestion, it seems apparent that copper concentrates in tissues rich in lipases and certain other enzymes. May we risk another step and say the copper is concentrated in the enzymes? Be this as it may, it was these considerations that led me to test the following commercial preparations:

A. *Pancreatin* (Merck's).

This preparation contained all the pancreatic enzymes. The ash from about .2 gr. gave a marked reaction for copper as copper xanthate.

B. *Pancreatin from Pig* (Squibb's).

Result exactly as in *A*.

C. *Pancreatin* (Parke-Davis).

Result as in *B* and *A*.

Is the copper associated with the lipases exclusively? This can not be determined from the above tests. I therefore tried a proteolytic enzyme likely to be free from lipases, amylases, and other enzymes.

D. *Pepsin* (origin unknown).

The ash from .2 gr. when freed from iron gave no positive indication for copper. However, the iron hydroxide was found to hold the copper back. I therefore washed the precipitated hydroxide with ammo-

nia, whereupon the xanthate gave a very faint though unmistakably positive test.

E. Pepsin (Parke-Davis).

Also positive; faint after precipitation of copper by iron hydroxide.

These tests prove that copper occurs in preparations of pancreatic enzymes and pepsin. The association with lipolysin, therefore, is not a necessarily isolated instance, but very possibly a special illustration of a general rule. Indeed, the studies of von Euler and Svanberg ('20[2]) definitely foreshadow a natural relation between enzymes and heavy metals.

IX. Localization of Copper in Eggs Exposed to Copper Solutions.

Normal eggs, in addition to secreting copper compounds, remove copper sulphate from sea-water when this salt is present. In eggs so exposed we should expect differences, if not in distribution, then at least in the concentration of the copper which they contain. Such analyses seem doubly worth while, for they seem not only to control and correct the observations on normal material, but may ultimately throw some light on the machinery of copper fixation and metabolism in general.

Tests consequently were made on material with a total volume of .5 c.c. exposed for 20 minutes to 5 c.c. of a 1/180 *n* Cu solution in sea-water. In every other respect the eggs were treated as described in section VII. Moreover, the material was from the same lot as that used for the localization of copper in normal eggs.

1. *Copper Absorbed by the Chorion.*

From the experiments of section IV. we expect for the jelly of exposed eggs a copper content greater than that found in the normal chorion. This expectation, of course, is realized, as can be shown by means of the hæmatoxylin test with entire eggs. Superficially such eggs take on an unmistakable blue color which microscopic examination localizes distinctly in the jelly. The same effect is gotten with the triple-nitrite test, only the color is then black instead of blue.

2. *Copper Absorbed by the Egg Membrane.*

With hæmatoxylin the membrane of the exposed egg is bluer than the normal. The triple-nitrite test, however, is more convincing. In optical sections of the surface one no longer finds individual black beads strung on a thin thread, but a heavy continuous black line. Even the highest powers fail to resolve the continuity. It seems very clear, indeed, that potassium-copper-lead nitrite is more abundant than in normal membranes.

On this basis it is possible to understand one of Lillie's results. If an excess of copper in the membrane alters it so that spermatozoa are not able to penetrate as easily as under normal conditions, we should expect "a certain virtue in mass action in the presence of this inhibitor of fertilization" (*loc. cit.*, p. 129). I noted exactly the same virtue ('15) in connection with eggs which had been exposed to calcium. This particular point and its meaning have both been overlooked in subsequent discussions.

3. *Copper Absorbed by the Cytoplasm.*

Inside the exposed egg there are differences in the same sense. The number of black granules after the nitrite treatment is greatly increased. As a whole and in sections the exposed egg is distinctly darker than the normal. Copper evidently passes inward through the membrane and diffuses generally through the cytoplasm.

4. *Copper Absorbed by the Cortex.*

If, as Lillie's experiments indicate, the cortex contains "unsaturated" copper-avid material, one may expect very considerable differences in the cortical zone. As a matter of fact, the triple-nitrite test brought to light black beads similar to those seen in and immediately under the normal membrane; they were decidedly more numerous than in normal eggs and gained in distinctness by heating. Furthermore, the blue zone which in normal eggs resists resolution by all the methods employed now differentiates slightly with both potassium ethyl xanthate and potassium ferrocyanide. With the first, the blue acquires a greenish tint and becomes separated from the egg membrane by an exceedingly narrow yet distinguishable yellowish layer; with the second, a very thin brown band intervenes between the original blue and the membrane.

These changes become intelligible if we assume, in the one case, the formation of minute traces of copper xanthate; in the second, of copper ferrocyanide. Since the same reagents produce no noticeable changes in these locations in untreated material, it appears that the cortex of an egg exposed to copper contains more than the normal quantity of the metal. Moreover, the excess is localized chiefly near the surface, and since it is not the agglutinin that is copper bearing, but the lipolysin, it appears probable that the latter is also concentrated immediately under the vitelline membrane.

X. Copper Map of the Normal Egg.

From all these tests—those on the controls as well as those on material exposed to copper sulphate, I have constructed a chart which indicates the distribution of copper in the normal *Arbacia* egg. This map is a visual summary of the chief results and inferences, and in view of the preceding discussion seems to require no other comment than that given in the legend under the figure.

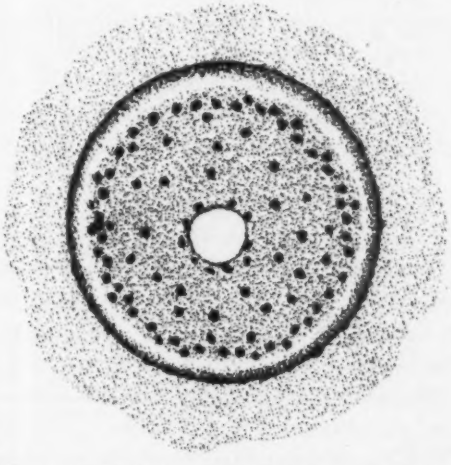

Diagram showing distribution of copper in the normal egg of *Arbacia punctulata*. The central clear area is the nucleus. Immediately about this, and extending to the cortex, the larger black spots represent pigment granules in which copper was demonstrated indirectly by analysis of secreted pigment and directly, in situ, as potassium-copper-lead nitrite. The latter, as well

as hæmatoxylin, indicates also the presence of diffuse copper in the cytoplasm. This is shown by the finer stipples.

The cortical layer is shown as differentiated into two zones: an inner, free from copper; an outer, immediately under the egg membrane with considerable concentration of copper. The evidence is again the triple-nitrite test on normal eggs, and the xanthate and ferrocyanide tests on eggs which had been exposed to solutions of copper in sea-water.

The vitelline membrane is shown as a line whose irregularly spaced black beads represent regions in which the triple nitrite was concentrated. The membranes of eggs exposed to copper solutions appear as continuous heavy black lines without beads.

The region outside the vitelline membrane represents the chorion with its diffuse and highly rarefied copper.

The diagram is intended to indicate distribution. It cannot indicate quantitative differences accurately because a variety of tests was used and the extent of discoloration depends not primarily on the quantities of copper but on the molecular size of the copper compounds formed. These are necessarily different in the several tests.

XI. Order of Magnitude of the Quantities of Copper Involved.

Quantitative data on the copper content can not be given. The methods for determining the copper are, indeed, reliable enough; but, unfortunately, the chorion alone prevents anyone from knowing how many eggs are present in 1 c.c., and until this number is definitely ascertained our initial measurements can hardly articulate closely with the results of chemical methods unusual in delicacy. For the present, then, we can arrive only at approximations.

1. *The Ovarian Egg.*

Eleven c.c. of ovary were dissolved in 11 c.c. of concentrated nitric acid and after complete destruction diluted to 100 c.c. with distilled water. Ten c.c. of this solution were evaporated to dryness and the incinerated ash dissolved. After neutralization with ammonia the volume was restored to 9 c.c. To this was added 1 c.c. of a solution of potassium ethyl xanthate whose concentration was 1 gr. per 1,000 c.c. of distilled water. This same amount was added to 9 c.c. of a copper sulphate solution containing 10 μ gr. of copper per c.c.

Colorimetric comparisons were then made, and the concentration, C_2, of the unknown calculated from the concentration, C_1, of the standard and the depth of the unknown, D_2, necessary to match

a given depth, D_1, of the standard. Accordingly,

$$C_2 = \frac{C_1 \times D_1}{D_2}.$$

Test	$\frac{C_1 \times D_1}{D_2}$	Micrograms Cu. per c.c. Eggs.
I.....	$\frac{10 \times .50}{2.21}$	20.54
I.....	$\frac{10 \times .30}{1.38}$	19.76
II.....	$\frac{10 \times 2}{1.40}$	14.29
II.....	$\frac{10 \times 2}{1.31}$	15.27
III.....	$\frac{10 \times 1.5}{1.12}$	13.39

Average = 16.65 μgr.

2. *The Shed Egg.*

Ten c.c. of the material referred to as *A* in section VI. were treated and tested in exactly the same way.

Test	$\frac{C_1 \times D_1}{D_2}$	Micrograms Cu. per c.c. Eggs.
I.....	$\frac{10 \times 1}{1.55}$	161.25
I.....	$\frac{10 \times 1.5}{2.2}$	170.50
II.....	$\frac{10 \times 1}{1.40}$	178.50
II.....	$\frac{10 \times 1}{1.33}$	188.00

Average = 174.56 μgr.

3. *The Fertilized Egg.*

Forty c.c. from a solution of 1.2 c.c. of fertilized eggs gave the following results:

Test	$\frac{C_1 \times D_1}{D_2}$	Micrograms Cu. per c.c. Eggs.
I.....	$\frac{10 \times .5}{2.00}$	20.83
I.....	$\frac{10 \times .5}{1.96}$	21.25
I.....	$\frac{10 \times .5}{2.03}$	20.50

Average = 20.86 μgr.

The copper content of the fertilized ovum apparently has the general order of magnitude characteristic of the unshed ovarian egg. That of the shed egg is, roughly, from eight to ten times greater.

XII. Discussion.

1. *The Copper Problem.*

Unless bound up in respiratory or other pigments, copper is considered essentially a poison. Its use in germicides and fungicidal solutions, the harmful influences on higher and lower plants, power to block organic catalysis, the medicinal properties of colloidal suspensions and copper salves—all support the prevalent view. Yet there are no poisons in nature; there are only poisonous effects. These may be exercised by the commonest articles of diet at certain concentrations and by copper, it happens, at very low ones.

Even a cursory examination of the literature suggests further misgivings. Copper has been found repeatedly in three of the five main divisions of the plant kingdom and among animals, with equal frequency, in nine phyla, ranging from protozoa—if *Volvox* ('21[6]) is an animal—to man. However, only Maquenne and Demoussy ('20[3]), who worked on plants, and Rose and Bodansky ('20[1]), whose studies cover a wide range of marine animals, are actually bold enough to suggest that copper in general may be more than an adventitious element which living things somehow tolerate.

Specific processes in which it normally plays a rôle are still unknown; moreover, they are quite likely to remain so unless we free ourselves for investigation by an admission of ignorance.

As a matter of fact, copper has many of the qualifications of a

biological element. It is widely diffused in the environment and has been so since earliest geological time. Though listed among the less active metals, it is capable of entering into a large variety of combinations, including numerous organic unions. It is difficult to see how a living thing could avoid copper except by some definite mechanism of exclusion.

The absence of this is significant, but does not establish physiological importance. Very possibly living things are mere sieves that hold the copper back; very possibly, too, its marked concentration in such tissues as the liver is a liability, by accident dependent on other attributes and under ordinary circumstances negligible. Still one would like to be certain, and in our present state of knowledge this is impossible. Yet a negative answer, excluding copper from the realm of physiological processes, even now appears unlikely. It is impossible to conceive the synthesis of respiratory pigments, turacin, or any other product without thinking of a long linkage of reactions inevitably affected, directly or otherwise, by their final end result.

2. *Copper and Enzymes.*

Von Euler and Svanberg (*loc. cit.*) have shown that the addition of saccharase results in a marked reduction of the free silver ions in dilute solutions of silver nitrate. Since the silver did not become colloidal under the influence of reductions, possibly due to certain constituents of the enzyme preparation, these writers suggest a binding of the silver ions to certain constituents of the saccharase solution.

Since such unions render the enzyme inactive, why not assume that the constituent of the solution with which the metallic ions combine is the enzyme itself, an organic co-enzyme, or both? Such an assumption seems all the more reasonable because prolonged dialysis, I find, does not remove all the copper. Thus if copper incapacitates at all, catalysis would be excluded in any case.[4]

[4] It is not possible that certain differences of proportionality between the effects of silver and mercury salts could be explained on this basis? Perhaps too the recovery known as "The Danysz Phenomenon" results from a redistribution of metallic ions between enzyme and co-enzyme. Even if co-enzyme and enzyme together make up, and are identical with, the entity, enzyme, the allocation of Cu to different positions in this system might have results essentially those suggested both in sense and in degree.

This assumption involves on the part of enzymes or organic coenzymes a capacity for combining with heavy metals. Since this power is not only very great, to judge by the dilutions that incapacitate, but likewise inseparable from normal ferments, we should not be astonished if enzymes from copper-bearing organisms or tissues give positive results when tested for copper.

3. *The Oligodynamic Effects of Copper.*

The poisonous effects of extraordinarily high dilutions of metallic copper were first noticed by Nägeli ('93), who found one part in seventy-seven millions rapidly fatal to *Spirogyra*. Because of the extremely small quantities involved Nägeli spoke of "oligodynamic action."

As these results have been repeatedly verified on other forms, it may be convenient to retain Nägeli's term. However, "oligodynamic" need imply nothing more mystical than a chemical or perhaps physical relation between extremely small quantities of material. As yet no acceptable explanation has been offered.[5]

But there have been suggestions. Nägeli was able to destroy the toxicity of oligodynamic solutions by means of paper, wool, paraffin, gums, and gelatin. The experience is quite comparable to the protective action of egg-water, gum arabic, and gelatin described by Lillie ('21[2]). Inasmuch as the copper in the first case at least is probably present as electropositive colloidal hydroxide or carbonate, Bayliss ('15[2]) imagines adsorption on the electronegative surfaces of Nägeli's detoxicants as the basis of their antitoxic properties. Conversely, the toxic effects become explicable as the outcome of adsorption by electronegative surfaces or particles critically involved in or on the affected tissues.

At this point Bayliss leaves the problem. We still do not know why such minute quantities are effective. Do they cause precipitation? If so, how much precipitation can they conceivably bring about? And, finally, what is precipitated? The neutralization of electrical charges in all likelihood is unavoidably associated with

[5] Quite recently, Saxl, as quoted by von Euler and Svanberg, assumed "dass die Metalle eine eigenartige Keimtötende Kraft besitzen, welche nicht mit der Löslichkeit der Metalle zusammen hängen soll." Saxl refers to a physical energy "die sich zunächst auf der Oberfläche der Metalle abspielt, jedoch auch in andere Medien übergehen kann" ('20[2] p. 378).

the ultimate mechanism of oligodynamic activity. For the present, however, our concern must be with facts a trifle more immediate. From these, it seems to me, we can derive a second suggestion.

The catalytic power of colloidal platinum is destroyed by traces of hydrocyanic acid and can be restored by simple aëration. Moore ('21[7]) compares this experience of Bredig and his pupils with the effects of hydrocyanic acid on the peroxidases of the enzymic oxidation system. "If these peroxidases are responsible in tissue cells for the uptake of oxygen by the protoplasm, it may well be that the poisonous action of hydrocyanic acid in such minute doses is due to interference with the action of the peroxidases" (*loc. cit.*, p. 231).

Disregarding the specific case which Moore had in mind, there is implicit here a definite relationship between enzymes and oligodynamic action. This relation, however, is also implicit in the copper inhibitions of Lillie. Again the union of saccharase with silver or copper implied by von Euler and Svanberg, together with the natural occurrence of copper in preparations of lipolysin, pancreatic enzymes, and pepsin, now reported, I believe, for the first time, are just what one might expect if the oligodynamic properties of heavy metals are traceable to the activation or inactivation of enzymes. These effects must depend on the capacity of enzymes to bind the metal. Until stoichiometrical determinations have been made nothing further can be said about such unions. But no matter what their nature may be, their occurrence should result in the presence of heavy metals in enzyme preparations whenever such presence is a physical possibility.

But the presence of copper raises an apparent difficulty. One might arrive at the paradox that enzymes, by the very nature of the case, must be normally incapacitated. This absurdity is easily dispelled. There is no reason for considering the normal enzyme as saturated with copper. For that matter, the enzyme proper may not be involved at all, for the observed effects can all be explained equally well if the copper is held by organic co-enzymes where these are present. But in this case also we are not compelled to consider the co-enzymes as saturated.

On this basis it is possible to explain the most puzzling feature of oligodynamic action. It is not that copper, silver, gold, or any other metals have this or that effect, but that infinitesimal amounts give results apparently out of all proportion to the quantities involved.

In the case of copper, for example, I imagine the oligodynamic effect as due to the inactivation of that fraction of the enzyme or co-enzyme which was not inactivated by the copper present in the first place. Where the oligodynamic effect is produced by silver or some other metal, the total of inactivated enzyme or co-enzyme would be composed of two fractions—the one inactivated by copper, the other by silver.

This view of the case has stoichiometrical implications which further work may or may not justify. But however this may turn out, the presence and effect of copper in preparations of normal enzymes calls urgently for further study.

Quite apart from the biochemical questions that suggest themselves at once, the discovery carries with it problems of wide biological significance. Is the liability to inactivation compensated by the production of larger quantities of the enzymes? Or does compensation come about by variety and differential susceptibilities? Why the tremendous number of enzymes when, aside from a few cases of molecular rearrangement, the only radical processes we have to deal with are the reversible oxidations and hydrolyses?

4. *Copper and Fertilization.*

Probably most of the copper present in the *Arbacia* egg is incorporated in the pigment whose elimination discolors the sea-water. As I have shown ('14), the rate of pigment secretion is increased 50 per cent. while the eggs are undergoing fertilization. This explains why the copper content of fertilized eggs is so much lower than that of unfertilized. My values for the copper content indicate in fertilized eggs an order of magnitude quite different from that in ripe eggs and essentially the same as that of the immature ovarian ovum. Tentatively, therefore, one may hazard that, among other things, fertilization restores the copper content to an order of magnitude characteristic of the unripe egg.

In the absence of more accurate data it is premature to discuss

this fact at length. Nevertheless we may possibly find along these lines some help in clearing up the uncertainties that now beset us. Very possibly the concentration of copper normally has something to do with checking the growth of the egg, whereas the heightened rate of secretion during fertilization restores conditions essential for further growth and development. No doubt the linkage can be pictured in several ways, but one way is this: the elimination of pigment from the egg might result in the production of fresh pigment, or some other product, which, if copper-avid, might also draw upon the copper of the enzymes and thus assist in the process of activation.

All this, however, is merely in the realm of possibilities. At the rate at which discoveries are being made in this field hardly anyone would wish to formulate a theory of fertilization. The process is far more complicated than it seemed ten years ago, and if one thing is more certain than another, it is that the major classes of evidence have not yet been handed in.

XIII. Summary.

1. Nearly 37 per cent. of the copper sulphate added to sea-water is precipitated at once when the concentration is Cu = $n/460$.

2. Two tenths c.c. normal *Arbacia* eggs in 75 minutes reduce the concentration of 14.8 c.c. of a Cu solution from $n/1{,}460$ to $n/1{,}790.9$.

3. In this reduction the egg jelly or chorion is heavily involved.

4. It was impossible to determine the quantities of copper absorbed by normal eggs and eggs without jelly because of an apparent secretion of copper by the eggs themselves.

5. Therefore, the demonstration of copper in *Arbacia* eggs was undertaken. The copper was identified as cupric hydroxide, cupric cyanide, cupric ferrocyanide, copper xanthate, crystalline cupric sulphate, as metallic copper on tin-foil and aluminum, and finally by electrolytic precipitation under conditions under which copper and only copper could be deposited.

6. The copper was localized in the egg directly by means of hæmatoxylin and the triple-nitrite of potassium-copper and lead. Indirectly it was localized by the analysis of egg secretions.

7. The copper occurs chiefly in the egg pigment; the vitelline

membrane and the chorion also contain copper. It is not a regular constituent of agglutinin precipitates, but was found constantly in precipitates of lipolysin.

8. This association with lipolysin makes possible the localization of both copper and ferment in the cortex of the egg.

9. The association of copper with lipolysin is not an isolated case. Copper was found also in preparations of pancreatic enzymes and pepsin.

10. In eggs exposed to a *n*/180 Cu solution for twenty minutes the copper is widely diffused through the cytoplasm and concentrated in the chorion, the vitelline membrane, and the cortex.

11. Approximately the amounts of copper normally present in 1 c.c. of *Arbacia* eggs are as follows:

Unripe ovarian eggs	= 17 μgr.
Ripe shed eggs	= 175 μgr.
Fertilized eggs	= 21 μgr.

12. From the preceding and other considerations it is suggested that copper, in general, may be more than an adventitious element, physiological only in pigments, and merely tolerated in all other connections.

13. The association of copper with enzymes is explained as the outcome of some sort of union, very likely chemical, between enzymes or co-enzymes, or both, and the metal. It is also suggested that possibly the differences in the proportionality between silver and mercury effects, as well as the Danysz recovery after "poisoning," may be due to the distribution and subsequent redistribution of the metallic ions between enzymes and co-enzymes.

14. The oligodynamic action of copper is explained as due to the inactivation of that fraction of the enzyme or co-enzyme which was not normally inactivated by the copper present in the first place. If inactivation is produced by silver, it is suggested that the total inactive enzyme or co-enzyme would be composed of two fractions—the one inactive because of the normal copper content, the other because of the silver added.

15. It is suggested that the concentration of copper in the ovum at maturity may have something to do with limiting the growth of the egg; that the elimination of copper-bearing pigment during

fertilization may indirectly restore or produce conditions essential for further growth and development.

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